Genetic engineering and lignin biosynthetic regulation in forest tree species

TANG Wei, Janet Ogbon, Aquilla McCoy
(North Carolina State University, Forest Biotechnology Group, Centennial Campus Box 7247, Raleigh, NC 27695-7247, USA)

Abstract: Genetic engineering of forest tree species is regarded as a strategy to reduce worldwide pressure on natural forests, to conserve genetic resources and ameliorate stress on global climate, and to meet growing demand for forest wood and timber products. Genetic engineering approaches toward the control or management of fungal pathogens, arthropod herbivores, bacterial and viral diseases, the use of pest resistance genes, and weed competitors are being studied. Although the production of transgenic trees is relatively recent and only a few species have been successfully genetically engineered in forest tree species, very useful and valuable information is available on the application of transgenic trees. Genes involved in important agricultural traits such as herbicide resistance, insect resistance, and wood quality have been isolated and have been used to genetically engineer trees. New technologies of plant molecular biology and genomics now make it possible high-efficient genetic improvement of forest trees. Genetic engineering promises to expand greatly the potential for genetic manipulation as new genes of commercial interest are discovered and utilized. Lignification is a process essential to the nature and evolution of vascular plants that is still poorly understood, even though it has been studied for more than a century. Recent studies on mutant and transgenic plants indicate that lignification may be far more flexible than previously realized. Pines with a mutation affecting the biosynthesis of the major lignin precursor, coniferyl alcohol, show a high level of an unusual subunit, dihydroconiferyl alcohol. It is also unusual as a plant polymer in that there are no plant enzymes for its degradation. These results have significant implications regarding the traditional definition of lignin, and highlight the need for a better understanding of the lignin precursor biosynthetic pathway. In this review, we describe the progress made recently in genetic engineering of forest tree species.

Key words: Transgenic trees; Genetic engineering; Lignification; Gene expression regulation
CLC Number: S718.46 **Document code:** A **Article ID:** 1007-662X (2001)02-0075-09

Introduction

The forest resource is extremely important for both developed and developing country's economy and significant investment is being made in the new biotechnology to increase overall managed forest productivity. Emphasis up until now has been on the development of routine techniques for the introduction of genes into tree species but research efforts are moving into the engineering of commercial traits such as those involved in wood quality and pest tolerance. Tree genetic engineering is dependent upon the availability of tissue culture protocols allowing whole plant regeneration from isolated pieces of tissues and on the availability of DNA transfer methods permitting the modification of the host genome. At present, tissue culture routes that are widely used are organogenesis and somatic embryogenesis. Organogenesis is the method of choice for species such as poplar and aspen and consists of plant regeneration through organ formation on an excised piece of plant tissue cultured on the proper tissue culture medium. Somatic embryogenesis is preferred to conifer species and it is a process mimicking zygotic embryogenesis. Structures similar to zygotic embryos, called somatic embryos, are formed on explants cultured on adequate tissue culture media. These methods allow the rapid multiplication in vitro of any particular genotype that can be submitted to genetic transformation procedures (Huang *et al.* 1991; Huang and Tauer 1998).

For application of genetic engineering to trees, two methods are used mostly depending on the species to be transformed (Charest and Michel 1991). The first method, which makes use of the property of the pathogenic bacterium *Agrobacterium tumefaciens* to transfer part of its DNA contained on a tumor inducing (Ti) plasmid to infected plant cells, is referred to *Agrobacterium*-mediated transformation and is used with angiosperm trees such as poplars and aspens for production of transgenic trees (Confalonieri *et al.* 1994; De Block 1990; Feuillet *et al.* 1995; Fillatti *et al.* 1987; Howe *et al.* 1994; Klopfenstein *et al.* 1991; Miranda Brasileiro 1991; 1992; Tsai *et al.* 1994). The second method, which is based on brute force to propel microprojectiles coated with DNA into a plant, is referred to as microprojectile-mediated DNA delivery and it is the only method that has up to now yielded reproducibly transgenic conifers (such as white spruce and black spruce) (Ellis *et al.* 1993; Charest and Duchesne 1995). The combination

Biography: TANG Wei (1964-) Research associate, Forest Biotechnology Group, Department of Forestry, North Carolina State University, Raleigh, NC 27695-7247, USA.
Received date 2000-12-15
Responsible editor: Chai Ruihai

of the proper tissue culture protocol with a suitable genetic transformation is essential for recovery of transgenic trees and introduction of genes involved in important silvicultural traits. As a general trend, gymnosperms are more difficult to engineer than angiosperm trees. Although genetic engineering of forest trees is still a new discipline and very little has been done on environmental impact assessment prior to the release of transgenic trees, most plant studies on environmental releases which have targeted crops (Burke *et al.* 1994; Kjellsson and Simonsen 1994) could be valuable to forest trees. These results will be especially useful for angiosperm trees such as poplar and aspen because all crop plants studied are angiosperms and fundamental biological similarities exist between these two classes of plants. Excellent transformation protocol and strategy (Fig. 1) are available for genetic engineering of gymnosperm forest tree species and angiosperm forest tree species (Birth 1997).

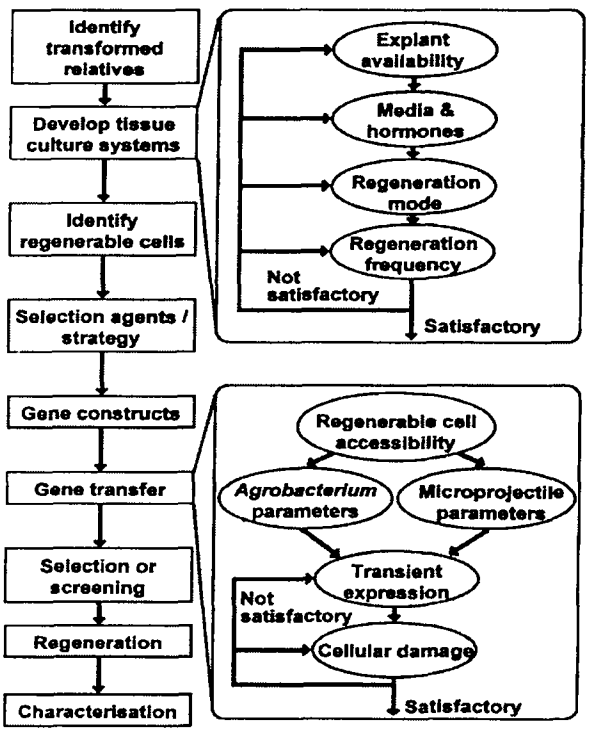

Fig. 1 Strategy to establishment of transformation systems for recalcitrant plant species

Lignin is conventionally defined as a complex hydrophobic network of phenylpropanoid units derived from the oxidative polymerization of one or more of three types of hydroxycinnamyl alcohol precursors. There are arguments in favor of an increased potential for genetic modification of lignin and indicate that our knowledge of the biosynthesis of lignin is far from complete. Lignin is unusual compared to other abundant natural polymers due to the low degree of order and the high degree of heterogeneity in its structure. Exploring the lignin biosynthetic pathway with genetic engineering technology can provide novel information to plant molecular biology and biochemistry community. In the present review, genetic engineering of gymnosperm forest tree species, genetic engineering of angiosperm forest tree species, interactions between transgenic trees and environments, and lignin biosynthetic regulation of forest tree species are discussed.

Genetic engineering of gymnosperm tree species

A successful genetic transformation method for gymnosperm forest tree species, especially conifers, is essential to access the improvements that can be achieved through genetic engineering technology. Study and use of potentially beneficial foreign genes in conifers is blocked because there is no efficient method for obtaining transformed plants from transformed conifer cells and tissues. DNA transfer using the biological vector *Agrobacterium* is still the most cost efficient and reliable method of transformation for plants. Transformation technology which uses a hypervirulent strain of *Agrobacterium* and the unique developmental properties of the shoot apical meristem for direct plant regeneration from the shoot apex, was recently developed, which has been used to obtain transformed plants of several recalcitrant crop species, and is adopted for conifers to transfer drought-inducible genes which have isolated from loblolly pine and salt brush. This will be useful to understand the role and function of these genes in conifers relative to drought tolerance, and to determine effectiveness of drought tolerance to commercial southern pine species.

Agrobacterium-mediated transformation method based on shoot apex inoculation is being used to produce transformed plants of loblolly and virginia pines. The *Agrobacterium tumefaciens* EHA101, containing the pTiBo542 virulence plasmid and the kanamycin sensitive derivative, were used. The influence of CaMV 35S promoter and the RbcS promoter that drive uidA (GUS) on gene expression efficiency is investigated. Plants of loblolly and virginia pines have been recovered and analyzed. Differential tissue specific GUS activity was conferred by these promoters indicative of genomic incorporation of uidA. Sequences unique to both transferred genes uidA and nptII were amplified by PCR in the DNA of recovered plants, and sequences specific to *Agrobacterium* (virG) were not amplified, indicating the plant DNA was not contaminated with *Agrobacterium* or plasmid DNA. Preliminary Southern blots of genomic DNA from recovered plants show bands with homology to uidA in high molecular weight DNA indicative of integration into a plant chromosome of stable transformation (Charest and Duchesne 1995).

Genetic engineering technology is related to gene isolation and genetic manipulation, genetic mechanisms underlying plant development and metabolism, and molecular defense mechanisms in plants. Large amount of researcher are conducting basic research to enhance our

understanding of biological processes in plants and to apply newly developing biotechnologies to facilitating genetic improvement or improving production systems to better utilize agricultural and natural resources. Research emphasis is to explore the molecular mechanisms controlling plant development and regeneration based on somatic embryogenesis and organogenesis. Currently, gene expression during cell differentiation and plant regeneration and identification and understanding of regulatory genes will allow us to develop better regeneration systems for plant regeneration in vitro. The engineering of reproductive sterility in trees is another valuable research area. Sterility in trees may result in increased productivity of wood as well as stopping dispersal of transgenes via pollen and seeds. An efficient transformation system for loblolly pine using gene gun and *Agrobacterium*, which will be used to study metabolic engineering of useful product biosynthesis for enhanced production through both cellular and molecular approaches, is being investigated. In addition, molecular defense mechanisms in trees and to identify novel genes and gene products will help plants defend themselves (Huang and Tauer 1998; Wenck *et al.* 1999).

Transformed *Pinus radiata* is a model of transformation of forest tree species. In New Zealand *Pinus radiata* D. Don, a conifer originally introduced from California, has been used for a century to meet some of these objectives, such as to produce large pruned and to use for pulp or reconstituted fiber products. Pine forests, which occupy only 4.5% of the land area of New Zealand, accounted for most of the domestic demand for forest products, as well as providing 13.5% of export earnings (NZ$ 2.6 billion) in the year to March 1994. This is a result of a climate which benefits tree growth, as well as innovative management and tree improvement practices. Conventional tree breeding technologies, such as control pollination, mass vegetative propagation from seedling stool beds, and micropropagation have been developed at NZ FRI and have been shown to have economic impact. Approximately 400 000 ha of the 1.3 million ha radiata pine estate in New Zealand is now established with genetically improved trees. Conventional breeding technology has led to the recombination of a wide spectrum of genes rather than specific alterations in only the trait of interest. Consequently, genetic improvement through molecular techniques has been considered for this species and other conifers. The New Zealand Forest Research Institute has been researching embryogenic tissue culture methods for *Pinus radiata* and other conifers for several years. The group, headed by Dale Smith, has been successful in developing reliable methods to regenerate plants by somatic embryogenesis from all families tested. Protocols for somatic embryogenesis of *Pinus radiata* developed have demonstrated the production of "seedlings" capable of normal growth under forest conditions. Experience over a number of years has shown that for control-pollinated families, on average 30%-35% of the megagametophyte explants will produce embryogenic cell lines. The best bryogenic cell lines. The best family gave an 87% response, the worst 13.6%. Between 26% and 32% of the embryogenic clones formed viable plants. Conversion of somatic embryos to plants ranged from 37% to 100%, with an overall average of 73% (Walter *et al.* 1998; 1999).

Transformation technology has been developed to introduce novel traits into clonal material, with the ultimate aim of making trees resistant to herbicides, insects, or other pathogens, and for introducing other desired traits. Transformation protocols using *Agrobacterium* or direct gene transfer methods have been established for various conifers, transgenic plants of *Pinus radiata* has been published (Walter *et al.* 1998; 1999). A molecular biology program which uses mapping and fingerprinting technology will ensure the genetic identity of the material and may allow identification of superior material on a molecular basis. In particle bombardment experiments, equal amounts of different expression constructs were coated onto gold particles. Significant differences in expression were observed, indicating a preference of the *Pinus radiata* expression system for certain controlling elements. Generally, the cloning of the Kozak consensus sequence around the ATG start codon increased the expression approximately 2.5-fold, regardless of whether the CaMV 35S or the double CaMV 35S promoter sequence was used. The Kozak sequence is regarded as an optimal binding site for the ribosome to start translation and our results show it is also effective in *Pinus radiata*. As in other plant transformation experiments, the double CaMV 35S promoter increased the expression strength over the single 35S promoter (Walter *et al.* 1998; 1999).

More than 40 geneticin resistant embryogenic lines were propagated for molecular analysis and plant regeneration. PCR results demonstrated the presence of the gus and the nptII reporter genes in those transformed cells, and embryos continued to grow and mature on selective media an expressed gus more than 8 months after transformation. Mature embryos from several separate transformation events using different clones of embryogenic tissue, were germinated and planted in soil. They are currently being grown-on for phenotypic analysis. Southern blot assays were carried out to confirm the integration of the introduced genes into the genome of *Pinus radiata* lines. One to five copies of the npt II gene were detected in the lines tested so far, and no tandem arrangements have been observed (Walter *et al.* 1998; 1999). The ability to transform and subsequently regenerate *Pinus radiata* has been demonstrated the capacity to introduce new traits into radiata pine as well as commence studies on developmental regulation of genes. In the future, the ability to alter the developmental regulation of genes and to influence developmental pathways may allow us to improve the yield of plantations through modification of cone development, wood formation, and other traits.

Genetic engineering of angiosperm forest tree species

Poplars are almost exclusively separate male and female trees and thus are potential for wide distribution of both pollen and seeds enable long distance gene dispersal. Therefore, poplars do not produce seed banks. In addition, competition from herbaceous weeds soon after germination precludes or greatly reduces survival. For aspens on upland or northern temperate to boreal sites, successful seedling establishment usually requires fire or a comparable intensive disturbance that exposes mineral soil and reduces competition. Because of the stringent conditions for reproduction by seed, vegetative reproduction is often more common than sexual reproduction for local dispersion. All poplars tend to sprout vigorously from stumps after trees are cut or fall from natural causes. Thus, genotypes can persist on sites for long time periods beyond the longevity of single trees (Mitton and Grant 1996). In addition, other tissues can serve as effective vegetative propagules. However, when wild stands are small compared to hybrid plantations, introgression may be observed after long periods of time, as has been detected at low levels among wild stands of *P. nigra* in Europe (Arens et al. 1998; Winfield *et al.* 1998).

The amenability of poplars to transformation via *Agrobacterium* (Han *et al.* 1996) and the possibility of map-based cloning because of their small genomes (Bradshaw 1996) make genetic engineering for pest resistance and other traits feasible. A large number of genome markers and marker technologies are available for genome analysis. Transgenic elite clones require limited field testing and can be rapidly deployed without further breeding to stabilize transgenic traits. There is likely to be strong resistance of wild poplar stands to significant introgression from plantations due to the combination of poplar traits such as delayed flowering, tree longevity, vegetative persistence, extensive wild stands, dilution of plantation-derived propagules by those from wild stands, stringent habitat requirements, and inability to establish under existing vegetation. Thus, except when a gene is employed that has a dramatic impact on tree fitness in the wild, the impacts of pest resistance genes are expected to be localized and slight for many decades. In the future, however, if transgenic trees become prominent in the landscape compared to wild stands and large areas become suitable for regeneration through natural or human causes, then genetic impacts could be more substantial and rapid. However, under near-term conditions, risk assessment and ecologically based analyses can focus on the consequences of new stands established very close to plantations and on the effects of numerically rare long-distance gene flow.

Disease is believed to be the most important factor limiting adoption and productivity of poplar plantations (Royle and Ostry 1995). Poplars are susceptible to many pathogens (Newcombe *et al.* 1996), and intensive culture has triggered changes in pathogen populations. Changes in North America have included the introduction of Eurasian pathogens (Newcombe *et al.* 1996), the movement of regional pathogens within North America (Newcombe 1998; Newcombe and Callan 1997), and hybridization between exotic and native species of the leaf rust pathogen Melampsora. Leaf rust is the most important disease of *Populus* worldwide. Host resistance has been the only widespread and economical control method for which both pathotype-specific and non-specific types of resistance are known (Newcombe *et al.* 1996). Exotic species of *Populus* are frequently resistant to native pathogens (Newcombe 1998), and resistance is often simply inherited in F1 interspecific hybrids. Genome analysis methods have allowed mapping of the genes for resistance to races E1, E2, and E3 (Cervera *et al.* 1996) and the Mmd1 gene for resistance to Melampsora medusae (Newcombe *et al.* 1996). The Mmd1 gene is expected to be physically isolated and transformed into a susceptible genotype in the near future, demonstrating the feasibility of genetically engineering disease resistance using native genes. The prospect of attempts to increase resistance using heterologous genes having broad and durable effectiveness against major pathogens, without negative effects on fitness, appears remote using current and foreseeable technology. These transgenes therefore do not appear to have the potential to significantly impact poplars in wild systems via introgression of transgenes. Moreover, transgenes that might give a useful degree of resistance in a well-tended genetic monoculture such as a clonal plantation are unlikely to be comparably important to pathogen resistance in genetically and environmentally diverse wild populations. Simple alterations in expression of native poplar or pathogen genes, such as by inducing constitutive overexpression or cosuppression, were also considered unlikely to be of significant ecological consequence. The transfer of unmodified resistance genes between *Populus* species is commonplace in conventional poplar breeding and should bring about similar risks if accomplished via gene isolation and genetic transformation. This should apply equally to leaf rust (Newcombe *et al.* 1996) and other diseases of *Populus*.

Insect damage is a major limitation to plantation viability and productivity in many regions (Strauss *et al.* 1998). Currently, the primary control method uses pesticides rather than resistant genotypes. Genetically based resistance is known but is often either incomplete or would require major alterations of breeding programs to accommodate, such as the use of different species as hybrid parents, with a consequent reduction in genetic improvement of other traits. The cottonwood leaf beetle is the major pest of poplars in the United States and is believed to be largely restricted to poplars and other species in the same family. The *cryIIIA* toxin from Bt (*Bacillus thuringiensis*) is highly toxic to some insects when applied topically or expressed in transgenic

poplars (Strauss *et al.* 1998). Lepidopter and efoliators are episodically significant, many have broad host ranges (e.g., gypsy moth and forest tent caterpillar), and most are sensitive to Cry1A Bt toxins (Kleiner *et al.* 1995). Wood borers of several taxa can be important pests in specific areas; because they are hard to be reached with topical pesticides, the use of transgenes could be an important control option. Insect damage in wild stands is sporadic in space and time though rarely results in genotypic mortality because of poplar resprouting capability. Thus, invasion of established stands by progeny of insect resistant transgenic trees is expected to be very slow. Field trials of transgenic poplars with beetle and caterpillar-active Bt transgenes are underway in several areas (Ellis and Raffa 1997; Leple *et al* 1992; Strauss *et al.* 1998), most notably China, France, and the northwestern United States. Other than Bt, work with alternative insect resistance transgenes has been limited. Proteinase inhibitors expressed in poplars have given either modest levels of resistance or none at all (Leple *et al.*1995; Confalonieri *et al.* 1988) and thus do not appear to be under consideration for commercial use. Genes with different modes of action, but as effective as Bt against poplar pests, are unknown.

The most important consideration when using Bt transgenes is the significant potential for development of Bt-resistant insect biotypes if the extensive transgenic poplar plantations are established without accompanying resistance management considerations (Raffa 1989). High levels of resistance have readily been bred in laboratory colonies under Cry3A selection. For most poplar plantations, wild stands are expected to provide large refugia that can slow resistance development and may obviate the need for planted refugia. However, the role of natural stands in the dispersal and mating behavior of target pests in areas where transgenic trees are being deployed should be studied. The working group considered that the potential for resistant biotype development from plantation use was a far greater concern than the risk of Bt transgenes providing a significant fitness advantage in wild trees after introgression. Sterility or other strategies for stringent gene containment were therefore not viewed as essential for use of pest resistance transgenes.

High levels of weed control for the first one to three years are essential for obtaining high rates of survival and tree growth in poplar plantations (Tuskan 1998). Plantation managers in many parts of the US believe that herbicide resistance (HR), particularly to glyphosate, can significantly reduce weed management costs and increase tree growth by providing more effective weed control and increasing moisture availability to trees. Because of the common use of poplars as windbreaks between agricultural fields and the future likelihood of their increased use for biofiltration plantings near streams in agricultural areas, HR poplars resistant to spray drift may be important components of agroecosystems dominated by glyphosate tolerant crops. Transgenic poplars with high levels of field resistance to glyphosate and phosphinothricin herbicides have been demonstrated in field trials (Strauss *et al.* 1998). If transgenes are allowed to spread via seed, sprouting of HR poplars could complicate their control (Strauss *et al.* 1995). In some systems, poplars are considered "mild" weeds; examples include perennial crops (e.g., conifers),rights of way, and drainage ditches. Spread of HR trees would remove certain herbicides as control options, which could be an important loss in systems that must rely on one or few herbicides for control. HR trees also may complicate plantation management in significant ways, such as making the" volunteers" from seed produced in flowering stands more difficult to control in regenerating stands and requiring use of other chemicals forkilling resprouts from stumps after harvest.

Poplars and other trees present substantial difficulties for extrapolating from small trials to large-scale effects for several reasons: 1) The scale of potential impact of transgenic poplars is large because of their extensive dispersal of pollen and seed; 2) Nearly all pre-commercial field trials do not permit trees to flower to avoid environmental release of transgenes, limiting opportunities for study of transgene movement and impact on a small scale; 3) Because of the large size of trees and the need to study their growth over several years, tests using trees are costly in space and time. As a result, most trials are smaller than is optimal for obtaining information relevant to commercial use and for assessing ecological impacts; trials are of shorter duration than commercial releases; 4) Significant impacts due to gene escape can accrue in poplars and other forest trees over multiple generations. Therefore, risk assessments are required that span decades to hundreds of years and use complex predictive models, which are necessarily speculative and imprecise.

Interactions between transgenic trees and environments

To insure the commercial use of transgenic trees, strong considerations must be given prior to potential effects of the release of transgenic trees into the environment. Information obtained with crop plants is not necessarily applicable to tree species and the two major factors that have to be taken into account are the long lifecycle and the presence of wild populations of commercial forest trees. The potential risks that have to be evaluated are: i. development of weediness in transgenic trees (in particular, with poplar and aspen species); ii. Gene transfer to wild populations (sometimes called genetic pollution); iii. emergence of new pests when insect resistant transgenic trees are deployed; iv. development of target pests resistant to engineered resistance mechanism in trees; v. deleterious effect on the ecosystem by disturbance of ecological chain; and, vi. genetic erosion of the wild population of trees due to uncontrolled gene flow from the engineered population.

The main difference with short-lived crop plants in evaluating the risks is the potentially more profound effect of long living transgenic trees on the environment. This has direct impact on increasing selective pressures on other organisms associated with transgenic trees. Although the risk evaluation is on the trait that has been genetically engineered, the deployment strategy will be a major factor on the potential environmental impact. Several strategies can be used to minimize or eliminate risks for large-scale release of transgenic trees (Raffa 1989): i. Plantation of mosaics of engineered and non-engineered trees; ii. Introduction of multiple resistance genes in the case of engineering for pest tolerance; iii. Integration with other silvicultural practices; iv. Temporal and tissue-specific expression of engineered characters; v. male, female or both flower sterility to contain gene flow to wild populations; and, vi. Monitoring of transgenic tree plantations and surrounding forests.

Although almost no directly relevant information is available for trees species, studies on gene flow in natural populations and on basic biology and ecology will contribute significantly to environmental impact assessments of transgenic trees. The Plant Protection Act and the Pest Control Product Act are applicable to transgenic trees presenting a potential danger to other plants (weediness) and to transgenic trees genetically engineered for pathogen resistance in Canada, respectively. Forest tree species have very different biology than most crop plants, the differences with crop plants common to both classes of trees are associated to their long life cycle and to the fact that they are rarely grown as monocultures. This implies that their interaction with the environment is more pronounced. Furthermore, most commercial trees grown are also present in natural ecosystems, meaning that gene flow can occur between artificial and natural populations of trees. Additionally, several species can cross-hybridize within the same genus, complicating the potential patterns of gene flow from transgenic trees. With gymnosperms, differences are related to their position in the evolutionary scale. These plants are considered ancestors of crop plants. Consequently, gene structure and function could be different in this class of plants but studies on gymnosperm genes published until now indicate that most genes are conserved with angiosperms genes showing a high degree of protein and DNA sequence homology. Another difference is that conifers have a complex reproductive cycle (up to 2 years for pines), which has implications related to monitoring and containment of transgenic trees.

With most of these species, only marker genes to confirm transformation were introduced into the host genome; however, traits such as insect resistance (McCown *et al.* 1991; Ellis *et al.* 1993), herbicide resistance (Fillatti *et al.* 1987; Miranda Brasileiro *et al.* 1992), bioremediation potential (Stomp *et al.* 1993), and modification of wood quality (Dwivedi *et al.* 1994; Feuillet *et al.* 1995) have been targeted. The genes for these traits were isolated from crop plants and other possibilities are being explored. Several groups now are working on the isolation and characterization of genes fromtrees that are involved in important silvicultural traits (Charest and Duchesne 1995). At present, different traits of forest trees which are engineered are: 1) Herbicide Resistance (Fillatti *et al.* 1987; Miranda Brasileiro *et al.* 1992); 2) Insect Pest Tolerance (Braun et al., 1991; Raman and Altman 1994); 3) Bacterial Disease Resistance (Casteels *et al.* 1989; Anzai *et al.* 1989); 4) Fungal Disease Resistance (Cornelissen and Melchers, 1993); 5) Viral Disease Resistance (Fitchen and Beachy, 1993); 6) Heavy Metal Resistance (Stomp *et al.* 1993); 7) Modified Growth (Klee and Romano, 1994; Kurioka *et al.* 1992); 8) Flower Sterility (Mariana *et al.* 1991; DeBlock and Debrouwer 1993); 9) Frost Tolerance (Georges *et al.* 1990); 10) Drought Tolerance (Bartels *et al.* 1992); 11) Wood Quality Modification (Dwivedi *et al.* 1994; Kajita *et al.*, 1994; Feuillet *et al.* 1995). At present, emphasis of tree genetic engineering is on insect pest tolerance, fungal disease tolerance, flower sterility, and wood quality modification. Flower sterility is of particular interest because it can be used as a containment measure for gene flow from transgenic trees. Applications of genetic engineering in forestry will undoubtedly occur first in intensively managed plantations (high-value forestry), in seed orchards and for horticultural species because of the investment required for the production of this elite material.

Lignin biosynthetic regulation of tree species

Forest trees are one of the world's most important natural resources. As crop plants, they are in the earliest stages of domestication, and much of our wood is still harvested from natural forests. Until now, the types of genetic changes that made possible the domestication of agricultural crops have not been possible for trees because hundreds or thousands of generations are required for selection. Lignin biosynthesis is very important in the growth, development, and wood formation in forest trees. Phydroxyphenyl, guaiacyl, and syringyl subunits are the precursors, and they are derived from phenylalanine by deamination, followed by hydroxylation of the aromatic ring, methylation, and the reduction of the terminal acidic group to an alcohol. These alcohols have long been thought to be the direct precursors for lignin (monolignols). The lignin precursors can radically couple at several sites with each other, or, more frequently, with the growing lignin oligomer, to produce a complex polymer with a variety of intermolecular linkages (Sederoff *et al.* 1999; Compbell and Sederoff 1996). At least 20 types of linkages have been described, and it is likely that many more are present in low proportions. Here, we focus on a mutation in the last step of the precursor pathway: the formation of the monolignol coniferyl alcohol from conifer aldehyde. This step is catalyzed by the enzyme cinnamyl alcohol dehydrogenase (CAD; EC 1.1.1.195) encoded by a single gene in loblolly

pine (Sederoff *et al.* 1999; Compbell and Sederoff 1996). The discovery of a recessive mutant allele of the cad gene, cad-n1, in loblolly pine has permitted the study of pines with severe deficiencies of CAD enzyme (Ralph *et al.* 1997). The secondary xylem (wood) in cad-n1 homozygous seedlings acquires a brown color, very distinct from the nearly white color of wild-type pine wood . CAD deficiency causes dramatic changes in the accumulation and nature of soluble phenolics; it also alters the structure of the lignin polymer that is deposited in the cell wall. The color and many of the changes in wood chemistry are similar to those observed in transgenic plants and in brown midrib mutants with suppressed CAD activity (Sederoff et al. 1999). It is believed that the observed variation in composition and structure of lignin is still best explained by variation of the monolignol precursors and their abundance in the lignifying zone. The plasticity in lignin composition reveals new potential that extends beyond the traditional monolignol pathway for modification of the polymer by genetic engineering. Structural information has long been used to guide the search for underlying mechanisms for important biological processes. The combination of current methods of structural chemistry, biochemistry, cell biology and genetics which will be valuable to elucidate the nature and origin of the lignin polymer.

Genetic engineering provides the opportunity to make such changes through gene transfer in a matter of years, rather than centuries. An antisense construct for a gene in the lignin pathway can greatly reduce lignin, increase cellulose, and dramatically stimulate growth was reported in transgenic aspen (Hu *et al.* 1999). Such changes could be important in tree domestication and could ultimately lead to trees that are profoundly different from their current undomesticated progenitors, because of fast-growing, low-lignin trees could provide significant practical benefits. Removal of lignin from the wood cell walls is the most energy intensive and environmentally damaging step in wood processing for pulp and paper, so reducing the lignin content in trees could provide both economic and environmental benefits. Even more importantly, any increase in efficiency that allows production of more wood and wood products from less land helps conserve natural forests and reduces the environmental impact of processing wood into pulp and paper. Tree growth and wood formation can be manipulated by modifying the biosynthesis of lignin and cell wall. Previous attempts to modify lignin by mutation or suppression of enzymes in the pathway for monolignol biosynthesis have resulted in striking changes in lignin composition, but usually with no effect on lignin content. The 4CL enzyme is also the phenylpropanoid precursor for flavonoid biosynthesis and constitutes a branch point between flavonoids and lignin. In the study on transgenic aspen, an antisense construct was expressed to downregulate the gene for the xylem form of 4CL, and demonstrated a profound effect on wood composition and tree growth: At 10 months of age, the transgenic aspens contained up to 45% less lignin and as much as 15% more cellulose than non-transgenic aspens; and the growth of the transgenic aspens was substantially enhanced. The composition of lignin and the cellular morphology were unchanged (Hu *et al.* 1999). These results raise interesting issues concerning the regulation of the lignin biosynthetic pathway and the formation of the secondary cell wall. Moreover, the transgenic trees show other changes in wood composition that reveal a link between the different biosynthetic pathways for the major components of the wood cell wall. The interrelatedness of these responses indicates important molecular interactions that have yet to be defined. Most intriguing is the enhanced growth of the transgenic trees. The fact that the extent of growth enhancement was not directly correlated with lignin content prompted the authors to propose that other pathways other than lignin biosynthesis may be involved. The absence of an explanation for the effect of reduced lignin on growth does not detract from its potential impact as a possible way for controlling growth and development in trees. These results further support the view that the potential for modifying wood properties through genetic engineering (Sederoff *et al.* 1999).

Conclusion

Although significant pogress has been made in genetic engineering of forest tree species, the most important research area identified was; 1) The continued acquisition of highly effective pest resistance genes; 2) Engineered sterility systems which would simplify scientific regulatory assessments; 3) Modification of lignin biosynthesis; 4) Reproductive biology, particularly rates of gene flow through pollen, seed, and vegetative spread; and 5) Avoid some important agronomic factors that would impact plantation management. Too little is known about the impacts of herbivores and diseases in wild populations, the nature and genetic variation of resistance mechanisms, and the way in which changes in resistance genetics might directly and indirectly affect species interactions and ecosystem processes. Information is needed on the broader impacts of transgenic trees to help society and government assess their socioeconomic and environmental importance. What degree of economic and environmental values are expected in the medium term on farm, landscape, and regional levels for trees with multiple functional transgenes such as herbicide resistance, insect resistance, sterility, and disease resistance. If these transgenic technologies are used wisely, the economies they provide farmers and industries may significantly increase the representation of forest trees in agroecosystems in place of annual crops, with multiple environmental benefits.

Acknowledgment

We thank Professor Ron Sederoff and Professor Yingchuan Tian for their help to our transformation investigation.

References

Anzai, H., Yoneyama, K., Yamaguchi, I. 1989. Transgenic tobacco resistant to a bacterial disease by the detoxification of a pathogenic toxin [J]. Mol. Gen. Genet., **219**: 492-494.

Arens, P., Coops, H., Jansen, J., and Vosman, B. 1988. Molecular genetic analysis of black poplar (*Populus nigra* L.) along Dutch rivers [J]. Molecular Ecology, 7:11-18.

Bartels, D., Hanke C., Schneider K., Michel D., Salamini F. 1992. Adesiccation-related Elip-like gene from the resurrection plant *Craterostigma plantagineum* is regulated by light and ABA. [J] Embo. J., **11**: 2771-2778.

Birch, R. G. 1997. Plant transformation: problems and strategies for practical application [J]. Annu. Rev. Plant Physiol. Plant Mol. Biol., **48**: 297–326.

Bradshaw, H. D. Jr. 1996. Molecular genetics of *Populus* [C]. In: Biology of *Populus* and its implication for management and conservation, eds. Stettler, R.F., Bradshaw, H.D. Jr, Heilman, P.E., and Hinckley, T.M., 183-199. National Research Council Canada. Ottawa, ON: NRC Research Press.

Braun, C.F., Jilka J.M., Hemenway C.L., Turner N.E. 1991. Interactions between plants, pathogens and insects: possibilities for engineering resistance [J]. Curr. Opinion Biotech., **2**: 193-198.

Burke, T.,Seidler R., Smith H. 1994. Ecological implications of transgenic plant release [J]. Mol. Ecol., **3**: 1-89.

Campbell, M.M., Sederoff, R.R. 1996 Variation in lignin content and composition. Plant Physiol, **110**: 3-13.

Casteels, P., Ampe, C., Jacobs, F., Vaeck, M., Tempst, P. 1989. Apidaecins: antibacterial peptides from honeybees [J]. Embo. J., **8**: 2387-2391.

Cervera, M.T., Gusmao, J., Steenackers, M., Peleman, J., Storme, V., Vanden Broeck, A., Van Montagu, M., and Boerjan, W. 1996. Identification of AFLP molecular markers for resistance against *Melampsora laricipopulina* in *Populus*. [J]. Theoretical and Applied Genetics, **93**: 733-737.

Charest, P.J., Duchesne L.C. 1995. Recent progress in forest biotechnology in Canada [R]. Petawawa National Forestry Institute, Inf. Rep. PI-X-120. 144 pp.

Charest, P.J., Michel. M.F. 1991. Basics of plant genetic engineering and its potential applications to tree species [R]. Petawawa National Forestry Institute, Inf. Rep. PI-X-104. 48 pp.

Confalonier,i M., Allegro, G., Balestrazzi, A., Fogher, C, and Delledonne, M. 1988. Regeneration of Populus nigra transgenic plants expressing a Kunitz proteinase inhibitor (KTi3) gene [J]. Molecular Breeding, **4**:137-145.

Confalonieri, M., Balestrazzi, A., Bisoffi, S. 1994. Genetic transformation of *Populus nigra* by *Agrobacterium tumefaciens* [J]. Plant Cell Rep., **13:** 256-261.

Cornelissen, B.J.C., Melchers, L.S. 1993. Strategies for control of fungal diseases with transgenic plants [J]. Plant Physiol., **101**: 709-712.

De Block, M. 1990. Factors influencing the tissue culture and the *Agrobacterium tumefaciens* mediated transformation of hybrid aspen and poplar clones [J]. Plant Physiol., **93**: 1110-1116.

De Block, M., Debrouwer, D. 1993. Engineered fertility control in transgenic *Brassica napus* L.: histochemical analysis of anther development [J]. Planta, **189**: 218-225.

Dwivedi, U.N., Campbell, W.H.,. Yu, J, Datla, R.S.S., Bugos, R.C., Chiang, V.L., Podila, G. K..1994. Modification of lignin biosynthesis in transgenic *Nicotiana* through expression of an antisense O-methyltransferase gene from *Populus* [J]. Plant Mol. Biol., **26**: 61-71.

Ellis, D.D., McCabe, D., McInnis, S., Ramachandran, R., Russell, D.R., Wallace, B.J.Martinell, K.M., Roberts, D.R., Raffa, K.F., Cown, B.H. 1993. Stable transformation of *Picea glauca* by particle acceleration [J]. BioTechnol.,**11**: 84-89.

Ellis, D.D. and Raffa, K.F. 1997. Expression of transgenic *Bacillus thuringiensis* delta-endotoxin in poplar [C]. In Micropropagation, genetic engineering, and molecular biology of Populus, eds. Klopfenstein, N.B., Chun, Y.W., Kim, M.S. and Ahuja, M.R. 178-186. US Department of Agriculture Forest Service, Rocky Mountain Forest and Range Experimental Station.

Feuillet, C., Lauvergeat, V., Deswarte, C., Pilate, G., Boudet, A. Grima-Tettenati, J. 1995. Tissue- and cell-specific expression of a cinnamyl alcohol dehydrogenase promoter in transgenic poplar plants [J]. Plant Mol. Biol., **27**: 651-667.

Fillatti, J.A., Sellmer, J., McCown, B., Haissig, B., Comai, L. 1987. *Agrobacterium* mediated transformation and regeneration of *Populus* [J]. Mol. Gen. Genet., **206**: 192-199.

Fitchen, J.H., Beachy, R.N. 1993. Genetically engineered protection against viruses in transgenic plants [J]. Ann. Rev. Microbiol., **47**: 739-763.

Georges, F., Saleem, M., Cutler, A.J. 1990. Design and cloning of a synthetic gene for the flounder antifreeze protein and its expression in plant cells [J]. Gene, **91**: 159-165.

Han, K.H., Gordon, M.P., and Strauss, S.H. 1996. Cellular and molecular biology of Agrobacterium-mediated transformation of plants and its application to genetic transformation of Populus [C]. In: Stettler, R.F. Bradshaw Jr, H.D. Heilman, P.E. and Hinckley, T.M. (eds), Biology of Populus and its implication for management and conservation, 201-222. National Research Council of Canada. Ottawa, ON: NRC Research Press.

Howes, G.T., Goldfarb, B., Strauss, S.H. 1994. *Agrobacterium*-mediated transformation of hybrid poplar suspension cultures and regeneration of transformed plants [J]. Plant Cell Tiss. Org. Cult., **36**: 59-71.

Hu, W.J, Harding, S.A., Lung, J., Popko, J.L., Ralph, J., Stokke, D.D., Tsai, C.J., Chiang, V.L. 1999. Repression of lignin biosynthesis promotes cellulose accumulation and growth in transgenic trees [J]. Nat. Biotechnol., **17**: 808–812.

Huang, Y. and Tauer, C.G. 1998, Genetic Transformation in Conifers. In: S. Puri (ed.), Tree Improvement: Applied Research and Technology Transfer. Oxford & IBH Co., India, pp 301-325.

Huang, Y., Diner, A.M. and Karnosky, D.F. 1991. Agrobacterium rhizogenes-mediated genetic transformation and regeneration of a conifer: *Larix deciduas* [J]. In Vitro Cellular & Developmental Biology, **27**: 201-207.

Kajita, S., Osakabe, K., Katayama, Y., Kawai, S., Matsumoto, Y., Hata, K., Morohoshi, N. 1994. *Agrobacterium*-mediated transformation of poplar using a disarmed binary vector and the

overexpression of a specific member of a family of poplar peroxidase genes in transgenic poplar cells [J]. Plant Sci., **103**: 231-239.

Kjellsson, G., Simonsen, V. 1994. Methods for risk assessment of transgenic plants. I. Competition, establishment and ecosystem effects [M]. Boston: Birkhauser Verlag, 214 pp.

Klee, H.J., Romano, C.P. 1994. The roles of phytohormones in development as studied in transgenic plants [J]. CRC Rev. Plant Sci., **13**: 311-324.

Kleiner K.W., Ellis, D.D., McCown, B.H., and Raffa, K.F. 1995. Field evaluation of transgenic poplar expressing a *Bacillus thuringiensis cry1A(a) d-endotoxin* gene against forest tent caterpillar (Lepidoptera: *Lasiocampidae*) and gypsy moth (*Lepidoptera: Lymantriidae*) following winter dormancy [J]. Environmental Entomology, **24**:1358-1364.

Klopfenstein, N.B., Shi, N.Q., McNabb, H.S., Hall, R.B., Hart, E.R.,Thornburg, R.W. 1991. Transgenic *Populus* hybrid expresses a wound-inducible potato proteinase inhibitor II-CAT gene fusion [J]. Can. J. For. Res., **21**: 1321-1328.

Kurioka, Y., Suzuki, Y., Kamada, H., Harada, H. 1992. Promotion of flowering and morphological alterations in *Atropa belladona* transformed with a CaMV35S-rolC chimeric gene of the Ri plasmid [J]. Plant Cell Rep., 12:1-6.

Leple, J.C., Bonadebottino, M., Augustin, S., Pilate, G., Letan, V.D., Delplanque, A., Cornu, D., and Jouanin, L. 1995. Toxicity to *Chrysomela tremulae* of transgenic poplars expressing a cysteine proteinase inhibitor [J]. Molecular Breeding, **1**: 319-328.

Leple, J.C., Miranda Brasileiro, A.C., Michel, M.F., Delmotte, F., Jouanin, L. 1992. Transgenic poplars: expression of chimeric genes using four different constructs [J]. Plant Cell Rep., **11**: 137-141.

Mariana, C., De Beuckeleer, M., Truettner, J., Leemans, J., Goldberg, R.B..1991. Induction of male sterility in plants by a chimaeric ribonuclease gene [J]. Nature, **347**: 737-741.

McCown, B.H., McCabe, D.E., Russell, D.R., Robinson, D.J., Barton, K.A., Raffa, K.F. 1991. Stable transformation of *Populus* and incorporation of pest resistance by electric discharge particle acceleration [J]. Plant Cell Rep., **9**: 590-594.

Miranda Brasileiro, A.C., Leple, J.C., Muzzin J., Ounnoughi, D.,. Michel, M.F., Jouanin, L. 1991. An alternative approach for gene transfer in trees using wild-type *Agrobacterium* strains [J]. Plant Mol. Biol., **17**: 441-452.

Miranda Brasileiro, A.C., Tourneur, C., Leple, J.C., Combes, V., Jouanin, L. 1992. Expression of the mutant *Arabidopsis thaliana* acetolactate synthase gene confers chlorsulfuron resistance to transgenic poplar plants [J]. Transgenic Res., **1**: 133-141.

Mitton, J.B., Grant, M.C. 1996. The natural history of quaking aspen [J]. BioScience, **46**: 25-31.

Newcombe, G. 1998. A review of exapted resistance to diseases of *Populus* [J]. European Journal of Forest Pathology, **28**: 209-216.

Newcombe, G. and Callan, B.E. 1997. First report of Marssonina brunnea f.sp. brunnea on hybrid poplar in the Pacific Northwest [J]. Plant Disease, **81**: 231.

Newcombe, G., Bradshaw, H.D. Jr, Chastagner, G.A., and Stettler, R.F. 1996. Amajor gene for resistance to Melampsora medusae f.sp. deltoidae in a hybrid poplar pedigree [J]. Phytopathology, **86**: 87-94.

Raffa, K.F. 1989. Genetic engineering of trees to enhance resistance to insects [J]. BioSci., **39**: 524-534.

Ralph, J., Mackay, J.J., Hatfield, R.D., O'Malley, D.M., Whetten, R.W., Sederoff, R.R. 1997. Abnormal lignin in a loblolly pine mutant [J]. Science, **277**: 235-239.

Raman, K.V., Altman, D.W. 1994. Biotechnology initiative to achieve plant pest and disease resistance [J]. Crop Protection, **13**: 591-596.

Royle, D.J. and Ostry, M.E. 1995. Disease and pest control in the bioenergy crops poplar and willow [J]. Biomass and Bioenergy, **9**: 69-79.

Sederoff, R.R., Mackay, J.J., Ralph, J., Hatfield, R.D. 1999. Unexpected variation in lignin [J]. Curr. Opin. Plant Biol., **2**: 145–152.

Stomp, A.M., Han, K.H., Wilbert, S., Gordon, M.P. 1993. Genetic improvement of tree species for remediation of hazardous wastes [J]. In Vitro Cell Dev. Biol., **29P**: 227-232.

Strauss, S.H., Rottmann, W.H., Brunner, A.M., Sheppard, L.A.. 1995. Genetic engineering of reproductive sterility in forest trees [J]. Mol. Breeding, **1**: 5-26.

Strauss, S.H., Meilan, R., DiFazio S., Mohamed, R., Brunner, A., Leonardi, S., Skinner, J., and Krutovskii, K. 1998. Tree Genetic Engineering Research Cooperative (TGERC) Annual Report: 1997-1998 [R]. Forest Research Laboratory, Oregon State University, Corvallis.

Tsai, C.H., Podila, G.K., Chiang, V.L. 1994. *Agrobacterium*-mediated transformation of quaking aspen (*Populus tremuloides*) and regeneration of transgenic plants [J]. Plant Cell Rep., **14**: 94-97.

Tuskan, G.A. 1998. Short-rotation forestry: what we know and what we need to know [J]. Biomass and Bioenergy, **14**: 307-315.

Walter, C., Grace, L.J., Wagner, A., White, D.W.R, Walden, A.R, Donaldson, S.S, Hinton, H., Gardner, R.C., Smith, D.R. 1998. Stable transformation and regeneration of transgenic plants of *Pinus radiata* D.Don [J], Plant Cell Rep., **17**: 460-469

Walter, C., Grace, L., Donaldson, S.S., Moody, J., Gemmell, J.E., van der Maos, S., Kvaalen, H., Lonneborg, A. 1999. An efficient Biolistic transformation protocol for *Picea abies* embryogenic tissue and regeneration of transgenic plants [J]. Can. J. For. Res., **29**: 1539-1546

Wenck, A.R., Quinn, M., Whetten, R.W., Pullman, G., Sederoff, R. 1999. High-efficiency *Agrobacterium*-mediated transformation of Norway spruce (*Picea abies*) and loblolly pine (*Pinus taeda*) [J]. Plant Mol. Biol., **39**: 407-416

Winfield, M.O., Arnold, G.M., Cooper, F., LeRay, M., White, J., Karp, A., and Edwards, K.J. 1998. A study of genetic diversity in *Populus nigra* subsp. betulifolia in the Upper Severn area of the UK using AFLP markers [J]. Molecular Ecology, **7**:3-10.